GC Now Separates All Natural Amino Acids

A general method for gas chromatographic analysis of the natural protein amino acids has been developed by Dr. C. W. Gehrke of the University of Missouri. The analysis can be done in less than one hour.

Dr. Gehrke and one of his graduate students, W. M. Lamkin, now at the University of Texas medical school, previously developed a method of preparing *n*-butyl-*N*-trifluoroacetyl esters of 18 amino acids for gas chromatographic analysis (C&EN, Sept. 14, 1964, page 62).

In this procedure, *n*-butyl esters are acylated with trifluoroacetic anhydride for 120 minutes in methylene chloride. This method gives poor chromatographic results for arginine (no peak at all) and tryptophan (two peaks). The later studies show that the lack of an arginine peak was due to the formation of a trifluoroacetate salt of the guanido group, which dissociates during temperature programing in the chromatograph. The two tryptophan peaks result from the singly and doubly substituted trifluoroacetyl derivatives of the *n*-butyl ester.

Dr. Gehrke told the First Midwest ACS Regional Meeting, at the University of Missouri, Kansas City, that he and D. Stalling achieved quantitative conversion of arginine and tryptophan to reproducible derivatives by sealed-tube acylation of the *n*-butyl ester at 150° C. for five minutes, thus shortening the acylation time by two hours.

Further work has shown that the sealed-tube acylation procedure gives good results with each of the amino acids singly and no breakdown occurs. Thus far, studies on mixtures with different functional groups have shown no interactions.

Dr. Gehrke and F. Shakrohki also studied column packing materials to find one which would separate derivatives of all the amino acids. No suitable single stationary phase was found, but the required separation was obtained with a mixed column of 0.75/0.25 weight % of DEGS/EGSS-X in a borosilicate glass column, with temperature programing.

Comparisons of results by Stein-Moore and gas-liquid chromatography on a mixture of nine amino acids gave excellent agreement with the actual values. Research is in progress on applications of this method to protein hydrolyzates and biological systems.

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Amino Acids Yield to Quantitative Gas Chromatography

n-Butyl N-trifluoroacetyl esters of amino acids offer compromise

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Biological Chemistry

n-Butyl *N*-trifluoroacetyl esters of amino acids look like good candidates for quantitative gas chromatography of amino acids. Yields from six representative amino acid esters are quantitative and reproducible peaks have been obtained for 18 amino acids, according to Dr. Charles W. Gehrke (project director) and Dr. W. M. Lamkin of the University of Missouri.

Gas chromatography is fast, accurate, and sensitive. It thus offers advantages over other chromatographic methods for analysis of amino acids. Several research groups are involved in studies of gas chromatographic analysis of amino acids—Dr. A. L. Meister at Tufts University and Dr. H. A. Saroff at the National Institutes of Health and their co-workers make up two such groups.

But amino acids can't be analyzed directly by gas chromatography because they are not volatile. They have to be converted to volatile derivatives. A number of investigators have developed derivatives, some of which have been useful in separations. But no one has studied these derivatives in sufficient detail—particularly the quantitative aspects of their preparation—to permit devising a general analytical method, according to the Missouri workers.

With a quantitative study in mind, and emphasis placed on finding the required reaction conditions for quantitatively converting amino acids to volatile derivatives, Dr. Lamkin, with Dr. Gehrke, first settled on the *N*-acyl esters after trying several other derivatives. Of the *N*-acyl esters, the *n*-butyl *N*-trifluoroacetyl esters in particular were selected because they provide a satisfactory compromise between volatility and ease of separation.

For most purposes, Dr. Lamkin says, the choice is a toss-up between methyl trifluoroacetyl esters and *n*-butyl trifluoroacetyl esters. However, methyl esters are much more volatile; serious losses occur in concentrating some of these esters prior to gas chromatography. The *n*-butyl esters, however, do not show such losses.

Early tests with the *n*-butyl *N*-trifluoroacetyl esters produced unexplained peaks on the chromatograph. The Missouri workers traced these to breakdown in the metal flash heater. Dropping the temperature reduced the extraneous peaks. Decomposition problems were eliminated when the samples were injected directly on the chromatographic column without using the flash heater. Thus thermal stability of the esters is adequate. The esters are also stable with respect to time. Recovery of derivatives standing for more than 90 hours is quantitative.

The esters can be prepared quantitatively by preparation of the methyl esters, followed by interesterification. This procedure involves making methyl esters by esterification in methanol at room temperature for 30 minutes. This is followed by interesterification in 1-butanol for 180 minutes at 90° C. HCl is used as esterification and interesterification catalyst. The *n*-butyl esters are then put into a methylene chloride solution of trifluoroacetic anhydride for 120 minutes for acylation. With this method, solubility problems of cystine and the basic amino acids in butanol are avoided. For cysteine, esterification and interesterification have to be carried out under nitrogen. It's essential that the derivatives be stored under anhydrous conditions to prevent hydrolysis of the acyl derivatives of hydroxy and phenolic groups.

This procedure gives unsatisfactory results for arginine and produces two peaks for tryptophan. For the other 18 protein amino acids, reproducibility of individual derivatives is good, while the chemistry for arginine and tryptophan requires further study.

The relative molar response (ratio of the area per mole of amino acid to the area per mole of glutamic acid) was determined for the 18 amino acids and tryptophan. Reproducibility of response for acids carried through the entire chemical and chromatographic procedure was good.

A mixture of six amino acids (selected to have many different functional groups present) yielded the same relative molar response after the chemical and chromatographic procedure as did their counterparts carried through the procedure individually. Thus no problems of interaction interfere with the analysis.

A procedure was developed by the Missouri scientists for synthesizing macro amounts of pure *n*-butyl *N*-trifluoroacetyl esters as standards. These reference standards were used to determine the yield of derivative for six representative amino acids; yields of above 96% were obtained in each case. Thus the accuracy of the method is good, the two chemists note.

Dr. Gehrke says that other members of the research team (David Stalling and Frank Shahrokhi) have demonstrated quantitative yields for 15 amino acids, and a good peak has been obtained for arginine. Special molecular distillation apparatus was required to purify the derivatives of some of the amino acids used as reference standards. The Missouri research team is continuing work on the reaction conditions required for arginine and tryptophan, and additional work is being done on chromatographically separating the derivatives.

Considerable progress has been made on separations with mixed columns and temperature programing. Dr. Gehrke says the objective is to obtain quantitative recovery and separation of the 20 protein amino acids in one hour or less.

Agreement of Molar Responses Shows No Interaction Problem In Analysis

Component	Mixture	Individual Acid	Recovery %
Leucine	**0.733**	**0.777**	**99**
Serine	**0.533**	**0.540**	**99**
Methionine	**0.718**	**0.696**	**103**
Glutamic acid	**1.00**	**1.00**	**100**
Tyrosine	**0.985**	**1.022**	**96**
Lysine	**0.714**	**0.715**	**100**

Table shows relative molar response for n-butyl N-trifluoroacetyl esters made from six individual amino acids and from a mixture of the six amino acids. Molar response is chromatogram area per mole of amino acid. Relative molar response is the molar response of the particular amino acid compared to 1, the arbitrary molar response for glutamic acid. Agreement of the two molar responses shows there is no problem of interaction in analysis of mixtures. Yields are about quantitative